

REVIEW

IGF-binding protein-4: biochemical characteristics and functional consequences

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Abstract

IGFs have multiple functions regarding cellular growth, survival and differentiation under different physiological and pathological conditions. IGF effects are modulated systemically and locally by six high-affinity IGF-binding proteins (IGFBP-1 to -6). Despite their structural similarity, each IGFBP has unique properties and exhibits specific functions. IGFBP-4, the smallest IGFBP, exists in both non-glycosylated and N-glycosylated forms in all biological fluids. It is expressed by a wide range of cell types and tissues, and its expression is regulated by different mechanisms in a cell type-specific manner.

IGFBP-4 binds IGF-I and IGF-II with similar affinities and inhibits their actions under almost all *in vitro* and *in vivo* conditions. In this review, we summarize the available data regarding the following aspects of IGFBP-4: genomic organization, protein structure–function relationship, expression and its regulation, as well as IGF-dependent and -independent actions. The biological significance of IGFBP-4 for reproductive physiology, bone formation, renal pathophysiology and cancer is discussed.

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Introduction

Insulin-like growth factors (IGF-I and IGF-II), two growth-promoting peptides, have both mitogenic and metabolic actions that are involved in growth, survival and differentiation of many cell types and tissues under different physiological and pathological situations (reviews: Cohick & Clemmons 1993, Stewart & Rotwein 1996). IGFs can act in both an endocrine and a paracrine/autocrine manner (reviews: Cohick & Clemmons 1993, Mohan *et al.* 1996, Stewart & Rotwein 1996, Butler & LeRoith 2001). Gene-targeting studies in mice have demonstrated that both IGF-I and IGF-II are essential for growth and development (Liu *et al.* 1993, Liu *et al.* 1998, Butler & LeRoith 2001).

The IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors (IGF-IR and IGF-IIR). Most of the actions of IGF-I and IGF-II are mediated by the IGF-IR, which is a transmembrane receptor with tyrosine kinase activity (reviews: LeRoith *et al.* 1995, LeRoith 2000, De Meyts & Whittaker 2002). IGF-IIR binds IGF-II with high affinity but interacts minimally with IGF-I (review: Bralke 1999). Gene-

targeting studies revealed that IGF-IIR is important for the control of embryonic growth, and for internalization and degradation of extracellular IGF-II (reviews: Bralke 1999, Hassan 2003); however, it is unclear whether this receptor is involved in IGF-II signaling. IGF-IIR is identical to the cation-independent mannose-6-phosphate (Man-6-P) receptor that is involved in transport of Man-6-P-bearing lysosomal enzymes from their sites of synthesis into an endosomal/pre-lysosomal compartment (reviews: Bralke 1999, Hassan 2003).

The IGFs in serum and other extracellular environments are bound to specific IGF-binding proteins (IGFBPs), which represent a family of six secreted proteins with a common domain organization. They all have an N-terminal domain with 12 conserved Cys residues, a C-terminal domain with six conserved Cys residues, and a central (L) domain with no Cys residues except in IGFBP-4 (review: Duan 2002). The N- and C-domains are at least 50% homologous among the six IGFBPs in a given species, and for each IGFBP there is roughly 80% homology among different vertebrate species. The L-domain shows little similarity between species (reviews: Rechler 1993, Kelley *et al.* 1996). Since the affinities of

IGFBPs for the IGFs are equal to or greater than those of the IGF receptors, several mechanisms have evolved which decrease IGFBP affinities and increase IGF bioavailability to the receptors. These mechanisms include phosphorylation, glycosylation, proteolysis and the adherence to the cell surface or extracellular matrix (ECM) (reviews: Jones & Clemmons 1995, Clemmons 1997).

Recently, several so-called IGFBP-related proteins (IGFBP-rPs) have been discovered, which exhibit structural homology to the N-terminal region of the classical IGFBPs, but have substantially lower affinities for IGFs (review: Hwa *et al.* 1999). The functional significance of the IGFBP-rPs for the IGF system is currently unclear.

IGFBPs have a plethora of functions. In addition to acting as carrier proteins, IGFBPs have been shown to inhibit or potentiate IGF actions. In serum and other biological fluids, IGFBPs modulate the endocrine actions of IGFs by regulating the bioavailability of IGFs for their receptors. IGFBPs are also expressed locally in a broad spectrum of tissues and act as autocrine/paracrine regulators of IGF effects. Furthermore, some IGFBPs have been demonstrated to have IGF-independent actions (reviews: Murphy 1998, Wetterau *et al.* 1999, Mohan & Baylink 2002).

Among the six IGFBPs, IGFBP-4 is the smallest and is unique in that it has been consistently shown to inhibit IGF actions (Wetterau *et al.* 1999). IGFBP-4 was first described on the basis of its ability to potently inhibit bone cell growth (Mohan *et al.* 1989) and follicle-stimulating hormone-stimulated steroid production of ovarian granulosa cells (Ui *et al.* 1989). The most likely mechanism is binding of secreted IGFs, preventing their interaction with IGF receptors (Mohan *et al.* 1995b). However, possible IGF-independent pathways of IGFBP-4 action have also been discussed (Singh *et al.* 1994, Perks *et al.* 1999, Wright *et al.* 2002).

In this review, we summarize the present knowledge of the genomic organization of the IGFBP-4 gene, structure-function relationships of IGFBP-4, IGFBP-4 expression and its regulation, as well as the IGF-dependent and -independent actions of IGFBP-4. The biological significance of IGFBP-4 is also discussed.

Genomic organization of the IGFBP-4 gene

The human *IGFBP4* gene is located on chromosome 17 (Allander *et al.* 1993) and spans about 15.3 kb (Zazzi *et al.* 1998). According to the mouse genome sequence determined so far, the mouse *Igfbp4* gene spans 11.3 kb on chromosome 11 (<http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=16010>). The rat *Igfbp4* gene spans at least 12 kb of genomic sequence (Gao *et al.* 1993). The genes for human IGFBP-4 (hIGFBP-4) and rat IGFBP-4 (rIGFBP-4) are composed of four exons separated by three introns, which give them an arrangement similar to the genes of the other IGFBPs except for

IGFBP-3 (Cubbage *et al.* 1990). The splice sites are highly conserved between human *IGFBP4* and rat *Igfbp4* genes, but the sizes of the introns vary slightly between the two species (Zazzi *et al.* 1998). Cell type-specific transcript sizes were documented in mouse cell lines which, when translated, suggest an additional non-IGF-binding variant present in mouse cells (Glantschnig *et al.* 1998).

Alignment of the published rat (Gao *et al.* 1993), human (Dai *et al.* 1997, Zazzi *et al.* 1998) and mouse (Glantschnig *et al.* 1998) IGFBP-4 promoter sequences revealed an overall high evolutionary conservation, but some promoter regions show less conservation and vary between the three species. It is interesting that the human sequence differs from rodent sequences by a 12 bp insertion upstream to the transcription initiation codon (Dai *et al.* 1997). The IGFBP-4 promoter possesses a typical TATA box and a CAAT box. Several potential regulatory elements, such as cAMP responsive elements, steroid responsive elements, AP-1-binding sites and Sp1-binding sites exist in the IGFBP-4 5'-flanking regions of the three species (Gao *et al.* 1993, Dai *et al.* 1997, Glantschnig *et al.* 1998, Zazzi *et al.* 1998). These *cis*-regulatory binding sites provide the targets for a variety of local and systemic factors such as cAMP, parathyroid hormone (PTH) and various ligands of the steroid hormone receptor superfamily (such as glucocorticoids, retinoic acid, triiodothyronine, vitamin D), to regulate the expression of IGFBP-4 as discussed below.

Several *Alu* repeat sequences are clustered in the proximity (upstream) of the human *IGFBP4* gene, with an average of one *Alu* sequence per kb (Zazzi *et al.* 1998), which is a higher frequency than the normal distribution in the human genome (Houck *et al.* 1979). This indicates that the *IGFBP4* gene is a hot spot for *Alu* integration. High-density *Alu* regions are often sites of genomic instability (Calabretta *et al.* 1982) and show a higher frequency of sequence polymorphism (Batzner & Deininger 2002). Apart from the *Alu* repeat sequences, several polymorphic microsatellites were found within the boundaries of the human *IGFBP4* gene (Zazzi *et al.* 1998). One of these was used as a marker to locate the hereditary breast-ovarian cancer gene (Tonin *et al.* 1993). Another highly polymorphic microsatellite was found in the first intron of the human *IGFBP4* gene (Zazzi *et al.* 1998).

A typical cleavage site for poly(A) was found at the 3'-end of the human *IGFBP4* gene; however, no conserved poly(A) addition signal was detected within the 30 bp upstream region. Nevertheless, within this region an AAAAAA and several AACAAA consensus sequences were found, which could form a degenerate poly(A) addition signal. The few described eukaryotic genes that do not contain a standard AAUAAA sequence are involved in alternative polyadenylation, but this does not seem to be the case for the human *IGFBP4* gene, since no variation in mRNA length has been reported and no alternate polyadenylation site was found within the *IGFBP4* gene (Zazzi *et al.* 1998).

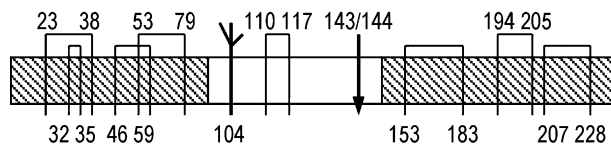


Figure 1 Structural features of hIGFBP-4. Numbers indicate cysteines involved in disulfide linkages (numbering based on the mature IGFBP-4 sequence derived from the proIGFBP-4 sequence containing a 21 aa signal peptide; SWISS-PROT accession number P22692). The conserved N-terminal and C-terminal regions of hIGFBP-4 are shaded, and the non-conserved midregion is unshaded. Cysteines are indicated by vertical lines and disulfide linkages by horizontal lines. The N-glycosylation site is shown by a trident and the PAPP-A cleavage site by an arrow.

The protein: structure–function relationship

hIGFBP-4 contains 237 amino acids (aa), and rIGFBP-4 consists of 233 aa. IGFBP-4 contains an N-linked glycosylation site and commonly exists in biological fluids as a doublet: a 24 kDa non-glycosylated form and a 28 kDa glycosylated form (Wetterau *et al.* 1999). IGFBP-4 is unique among the six IGFBPs in having two extra Cys residues in the variable L-domain encoded by exon 2 (Landale *et al.* 1995), which are linked to each other (Fig. 1) (Chelius *et al.* 2001). These unique properties of IGFBP-4 may be responsible for the distinctive biological behavior of this binding protein, i.e. solely inhibitory actions and lack of cell surface association.

IGF binding

IGFBP-4 inhibits IGF actions by preventing the binding of IGFs to their receptors. The binding of IGFBP-4 to IGFs is essential for this inhibitory effect (Mohan *et al.* 1995b, Miyakoshi *et al.* 1999). The IGF-binding domain may represent the major structural determinant of the biological activity of IGFBP-4. Therefore, elucidation of the structural determinants of various IGFBPs in IGF binding is important to the general understanding of the biology of the IGF system and may shed light on how these different IGFBPs exhibit specific actions.

It is generally accepted that the IGF-binding site for the various high-affinity IGFBPs is located in the N-terminal region. Mutational analysis indicated that the IGF-binding activity of hIGFBP-4 is mainly determined by the N-terminal region Leu⁷²–Ser⁹¹, and to a lesser extent by the C-terminal region Cys²⁰⁵–Val²¹⁴ (Qin *et al.* 1998). Although the three-dimensional structure of most IGFBPs has not been determined, disulfide bridging in IGFBPs appears to be important for maintaining the secondary structure required for IGF binding, since all six IGFBPs contain conserved Cys residues in both the N-terminal and the C-terminal regions and reduced IGFBPs exhibit little or no IGF-binding activity (Landale *et al.* 1995, Qin *et al.* 1998, Neumann & Bach 1999). Direct evidence

was provided by disruption of the disulfide linkages in the N-domain of rIGFBP-3, which resulted in complete loss of IGF-binding ability (Hashimoto *et al.* 1997). There is evidence suggesting that the IGF-binding domain in hIGFBP-4 involves a hydrophobic motif (Leu⁷²–Met⁸⁰) located in the distal part of the conserved N-terminal region, and that the N-terminal Cys residues (Cys9 and Cys12) are more critical than the C-terminal Cys residues (Cys17 and Cys20) for IGF binding (Byun *et al.* 2001a). Eight disulfide linkages in rIGFBP-4 and hIGFBP-4 have been determined, four in the N-terminal (two of them are present in all six IGFBPs), three in the C-terminal (present in IGFBP-2 and -6 as well) and one in the midregion (Fig. 1) (Chelius *et al.* 2001).

Although the IGF binding is mainly determined by the N-terminal conserved Cys residues, the six conserved C-terminal Cys residues in IGFBP-4 are essential for high-affinity binding of IGFs (Qin *et al.* 1998, Standker *et al.* 2000, Byun *et al.* 2001a). There is also evidence that in IGFBP-3 (Spencer & Chan 1995) and IGFBP-2 (Forbes *et al.* 1998, Wang *et al.* 1988) the C-terminal region plays an important role in IGF binding. The six C-terminal Cys residues in IGFBP-4 are linked in the same manner as in IGFBP-2 and -6 (Chelius *et al.* 2001). Both IGFBP-2 and -6 share a binding preference for IGF-II and have the same C-terminal disulfide linkages, suggesting that a different disulfide linkage could conceivably influence IGF-II binding preference (Forbes *et al.* 1998). However, IGFBP-4 binds IGF-I and IGF-II with similar affinities; thus the highly conserved three C-terminal disulfide linkages either have no effect on the IGF-binding preference, or they are not the sole determinants.

Cell surface association and tissue distribution

Cell surface and ECM association is one of the possible mechanisms to alter the affinity of IGFBPs for the IGFs. Both IGFBP-1 and IGFBP-2 have an Arg–Gly–Asp (RGD) motif, which was shown to bind $\alpha 5 \beta 1$ integrin and consequently to mediate cell surface association of IGFBP-1 (Jones *et al.* 1993). We demonstrated that IGFBP-2 bound to the plasma membrane also in the absence of the RGD motif in IGFBP-2 (Hoefflich *et al.* 2002), indicating that additional mechanisms are involved in cell surface association of IGFBP-2. IGFBP-2 can bind to heparin, ECM and proteoglycans depending on the previous binding to IGF molecules (Arai *et al.* 1996, Russo *et al.* 1999). IGFBP-3 and -5 bind to distinct membrane receptors (Oh *et al.* 1993, Andress 1995, 1998, Leal *et al.* 1997). However, there is no evidence for cell surface association of IGFBP-4 (Kelley *et al.* 1996), suggesting that IGFBP-4 exists primarily in a soluble extracellular form.

When IGFBP-3 was perfused through the isolated, beating rat heart, it crossed the microvascular endothelium

and was distributed primarily in cardiac muscle. In contrast, perfused IGFBP-4 also crossed the microvascular endothelium of the rat heart, but was preferentially distributed in connective tissue (Bar *et al.* 1990, Boes *et al.* 1992). A small basic C-terminal region (heparin-binding domain, HBD) of IGFBP-3 has been shown to be central to the ability of IGFBP-3 to bind to specific cells, such as endothelial cells (Booth *et al.* 1995, Knudtson *et al.* 2001). When this region was synthesized as an 18-mer peptide (P3), P3 bound to endothelial cells (Knudtson *et al.* 2001). IGFBP-4 lacks such an HBD and does not bind to endothelial cells (Booth *et al.* 1995). When the 20 C-terminal aa of IGFBP-4 region (P4) were replaced by the homologous P3 peptide, the generated chimeric IGFBP-4₃ bound specifically to endothelial cells, and it was distributed in the perfused rat heart similarly to the behavior of IGFBP-3 but different from that of IGFBP-4 (Knudtson *et al.* 2001), suggesting that the C-terminal region of IGFBP-4 is critical for its specific tissue distribution in the rat heart. These findings provide a novel potential mechanism of the tissue-specific actions of the IGFBPs.

Glycosylation

In 1991, Ceda and colleagues isolated two IGFBPs with apparent molecular weights (MW) of 28 and 24 kDa from the conditioned medium of B104 rat neuroblastoma cells (Ceda *et al.* 1991). Sequence analysis revealed that both proteins had identical N-terminal sequences and appeared to be two forms of IGFBP-4. Treatment of these IGFBPs with endoglycosidase-F reduced the MW of the 28 kDa IGFBP to 24 kDa. However, there was no change in the 24 kDa IGFBP. The data from this and other studies (Cheung *et al.* 1991, Carr *et al.* 1994) demonstrated that IGFBP-4 exists as both N-glycosylated and non-glycosylated protein. Further analysis revealed that a single N-linked glycosylation site is located in the midregion (Asn¹⁰⁴) of hIGFBP-4 and rIGFBP-4 (Fig. 1) and that the glycosylation of IGFBP-4 does not affect its binding to IGFs (Chelius *et al.* 2001). Five different glycosylation isoforms of rIGFBP-4, isolated from rat serum, were recently identified (Chelius *et al.* 2002). All identified oligosaccharides are bi-antennary and differ only in the number of sialic acid terminal residues and/or core modification with fucose. The physiological significance of the glycosylation in IGFBP-4 is unknown.

Proteolysis

Proteolysis is a major regulatory mechanism of IGFBP-4 functions. Each of the six IGFBPs can undergo proteolysis, which results in decreased affinity for IGFs. While some of the IGFBP proteases can use multiple IGFBPs as substrate, there are apparently proteases that are specific for individual IGFBPs (reviews: Maile & Holly 1999, Schneider

et al. 2002). An IGF-dependent IGFBP-4-specific protease was first reported in the media conditioned by both human and sheep dermal fibroblasts (Fowlkes & Freemark 1992), which was then identified as pregnancy-associated plasma protein-A (PAPP-A) (Lawrence *et al.* 1999b). This proteolytic activity has also been detected in the conditioned media from human osteoblasts (X Qin *et al.* 1999), vascular smooth muscle cells (Bayes-Genis *et al.* 2001), granulosa cells (Conover *et al.* 2001), trophoblast and decidualized endometrial stromal cells (Giudice *et al.* 2002), as well as in ovarian follicular fluid (Conover *et al.* 1999) and human pregnancy serum (Byun *et al.* 2001b).

PAPP-A was first isolated from human pregnancy serum (Lin *et al.* 1974) and belongs to the large metzincin family of metalloproteases (Lawrence *et al.* 1999a, Boldt *et al.* 2001). It cleaves IGFBP-4 at a single site, between Met¹³⁵/Lys¹³⁶ in hIGFBP-4 (Fig. 1) (Byun *et al.* 2000, Laursen *et al.* 2002a). IGFBP-4 cleavage by PAPP-A uniquely depends on the presence of IGF (Byun *et al.* 2000, Qin *et al.* 2000, Laursen *et al.* 2001). Recent data suggest that IGFs enhance the proteolysis by binding to IGFBP-4, but not by interaction with PAPP-A (Qin *et al.* 2000, Laursen *et al.* 2001). PAPP-A also cleaves hIGFBP-5 between Ser¹⁴³/Lys¹⁴⁴, which does not require the presence of IGF, but is slightly inhibited by IGF (Laursen *et al.* 2001, Rivera & Fortune 2003). PAPP-A can cleave bovine IGFBP-2 between Gln¹⁶⁵/Met¹⁶⁶ as well, and this proteolytic activity was shown to be dose-dependently enhanced by IGFs (Monget *et al.* 2003). PAPP-A is secreted as a dimer of 400 kDa, but exists in human pregnancy serum as a 500 kDa covalent heterotetrameric 2:2 complex with the proform of eosinophil major basic protein (proMBP), which functions as an inhibitor of the proteolytic activity of PAPP-A (Overgaard *et al.* 2000). The proMBP was also expressed in human fibroblasts after phorbol ester tumor promoter treatment and simian virus 40 transformation, and inhibited the proteolytic activity of IGFBP-4 (Chen *et al.* 2002). IGFBP-3, -5 and -6 can also function as inhibitors of IGFBP-4 proteolysis, probably through the homologous HBD in the C-termini of these IGFBPs (Fowlkes *et al.* 1997). A peptide derived from the HBD of IGFBP-5 (P5) is also able to inhibit the cleavage of IGFBP-2 by PAPP-A, and this inhibition is due to a direct interaction of P5 with PAPP-A rather than with IGFBP-2 (Monget *et al.* 2003). Recent data demonstrate that PAPP-A reversibly binds to the cell surface of several cell types, which does not affect the proteolytic activity of PAPP-A (Laursen *et al.* 2002b). This suggests that adhesion to the cell surface functions to target PAPP-A activity to the vicinity of the IGF receptor, decreasing the probability that released IGF is captured by other IGFBPs before receptor binding. Proteolysis of IGFBP-4 by PAPP-A enhances IGF bioavailability. Its physiological significance will be discussed below.

Table 1 Regulatory mechanisms for IGFBP-4 expression *in vivo*

	Tissue type/species	Effect	Reference
Agent			
Clenbuterol	Soleus muscle/r	s	Awede <i>et al.</i> 2002
EB1089	Prostate/r	s	Nickerson & Huynh 1999
Estrogen	Bone/m	s	Lindberg <i>et al.</i> 2002
	Serum/constitutionally tall girls	s	Rooman <i>et al.</i> 2002
FSH	Ovary/r	s	Putowski <i>et al.</i> 1997
GH	Serum/zinc-deprived rat	s	Ninh <i>et al.</i> 1998
	Serum/postmenopausal woman	s	Kassem <i>et al.</i> 1998
	Serum/bGH transgenic mice	s	Blackburn <i>et al.</i> 1997
GHRA	Serum, liver/m	s	van Neck <i>et al.</i> 2000
GHRP-2	Plasma/b (high feed intake)	s	Lee <i>et al.</i> 2000
	Plasma/b (low feed intake)	n	Lee <i>et al.</i> 2000
hCG	Mammary gland/r	i	Huynh 1998
	Ovary/r	s	Putowski <i>et al.</i> 1997
LPS	Serum/r	s	Soto <i>et al.</i> 1998
Nandrolone	Diaphragm muscle/r	i	Lewis <i>et al.</i> 2002
PMSG	Ovary/r	s	Putowski <i>et al.</i> 1997
T ₃	Kidney, serum/hypothyroid rat	s	Voci <i>et al.</i> 2001
	Liver, serum/hypothyroid rat	s	Demori <i>et al.</i> 1997b
	Uterus/r	s	Bottazzi <i>et al.</i> 1996
T ₄	Liver, mammary gland/r	s	Rosato <i>et al.</i> 2002

EB1089, vitamin D₃ analogue; FSH, follicle-stimulating hormone; GH, growth hormone; GHRA, GH receptor antagonist; GHRP-2, GH-releasing peptide-2; hCG, human chorionic gonadotropin; LPS, lipopolysaccharide; PMSG, pregnant mare's serum gonadotropin; T₃, triiodothyronine; T₄, thyroxine; b, bovine; h, human; m, mouse; r, rat; s, stimulation; i, inhibition; n, no effect.

IGFBP-4 expression *in vivo* and its regulation

IGFBP-4 has been identified in all biological fluids, including serum, follicular fluid, seminal fluid, interstitial fluid and synovial fluid (Rajaram *et al.* 1997). It is the second most abundant IGFBP in adult rat serum after IGFBP-3. Northern blot analysis revealed that IGFBP-4 mRNA is widely expressed in adult rat tissues, including adrenal gland, testis, spleen, heart, liver, lung, kidney, stomach, hypothalamus and brain cortex, with liver being the site of the highest expression (Shimasaki *et al.* 1990). The expression of IGFBP-4 was also examined in rat small intestine (Shoubridge *et al.* 2001), smooth muscle (Smith *et al.* 2001), skeletal muscle (Jennische & Hall 2000), pancreas (Hill *et al.* 1999), uterus and placenta (Cerro & Pintar 1997), mouse spinal cord (Arnold *et al.* 2000), mouse and human thymus (Li *et al.* 1996), human prostate (Thomas *et al.* 2000), bone (Mohan *et al.* 1995a) and ovary (Zhou & Bondy 1993, el Roeiy *et al.* 1994) of several species. In the mouse embryo, IGFBP-4 transcripts were detected as early as 11 days postcoitum (dpc) in different regions, including telencephalon, mesencephalon, snout, tongue and differentiating sclerotomes. After 14 dpc IGFBP-4 mRNA was undetected in the brain, but clearly detectable in lung, liver, kidney, intestine, vertebrae, ribs and incisivi (Cerro *et al.* 1993, Schuller *et al.* 1993). IGFBP-4 protein was localized in telencephalon, mesencephalon, heart, liver, lung, tongue, blood vessels and kidney of 13.5 dpc mouse embryo by immunohistochemi-

cal analysis (van Kleffens *et al.* 1999). These findings suggest that IGFBP-4 expression is developmentally regulated. Moreover, hormones, cytokines and other agents regulate the expression of IGFBP-4 in a tissue-specific manner (see Table 1).

IGFBP-4 expression *in vitro* and its regulation

Consistent with the widespread expression of IGFBP-4 *in vivo*, IGFBP-4 is expressed by various cell types *in vitro*, including fibroblasts, osteoblasts, myoblasts, epithelial cells, endothelial cells, chondrocytes and many kinds of tumor cells. The expression of IGFBP-4 *in vitro* is regulated by a large number of agents in a cell-specific manner. The effects of these agents are summarized in Table 2.

Actions of IGFBP-4

Several lines of evidence suggest that IGFBP-4 functions as a purely inhibitory protein *in vitro* and *in vivo*. These inhibitory actions can be exerted via IGF-dependent and -independent pathways (Fig. 2).

IGF-dependent actions

IGFBP-4 inhibits IGF-induced cell proliferation and differentiation in all cell types studied *in vitro* so far, including bone cells (Schiltz *et al.* 1993, Mohan *et al.* 1995b, Mohan & Baylink 2002), muscle cells (Damon *et al.* 1998a, Ewton

Table 2 Regulatory mechanisms for IGFBP-4 expression *in vitro*

	Cell types/species	Effect	Reference
Agent			
ACTH	Adrenocortical cells/h	n	Fottner <i>et al.</i> 2001
ACTH and IGFs	Adrenocortical cells/h	s	Fottner <i>et al.</i> 2001
Activin	Granulosa cells/r	i	Choi <i>et al.</i> 1997
Androgen	Osteoblasts/h	i	Gori <i>et al.</i> 1999
	Granulosa cells/h	n	Greisen <i>et al.</i> 2002
Angiotensin II	Vascular smooth muscle cells/r	i	Gustafsson <i>et al.</i> 1999b; Anwar <i>et al.</i> 2000
bFGF	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
	Fetal lung fibroblasts/r	s	Price 1999
cAMP	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
	Vascular smooth muscle cells/h	s	Hayford <i>et al.</i> 1998
	Osteoblasts/r	s	Chen <i>et al.</i> 1998
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	Articular chondrocytes/h	i	Di Battista <i>et al.</i> 1997
Dexamethasone	Bone marrow stromal cells/r	s	Milne <i>et al.</i> 2001
	Bone marrow stromal cells/h	s	Cheng <i>et al.</i> 1998
	Vascular smooth muscle cells/h	s	Hayford <i>et al.</i> 1998
	TC3 pancreatic beta cells	s	Katz <i>et al.</i> 1997
	Fibroblasts/h,b	i	Conover <i>et al.</i> 1995
EGF	Fetal lung fibroblasts/r	s	Price 1999
Estrogen	MCF-7 breast cancer cells/h	s	C Qin <i>et al.</i> 1999
	SaOS-2 osteoblasts/h	i	Kudo <i>et al.</i> 1996, 1997
	hFOB/ER9 osteoblasts/h	s	Kassem <i>et al.</i> 1996
FSH	Granulosa cells/r	i	Piferrer <i>et al.</i> 1997
GH	TC3 pancreatic beta cells	n	Katz <i>et al.</i> 1997
Glucagon	Ovarian thecal cells/b	n	Chamberlain & Spicer 2001
Glucose	Retinal endothelial cells/h	n	Giannini <i>et al.</i> 2001
IGF-I	Retinal endothelial cells/h	i	Giannini <i>et al.</i> 2001
	Glomerular endothelial cells/h	i	Giannini <i>et al.</i> 1999
	Uterine myometrial cells	s	Huynh 2000
	Smooth muscle cells/h	i	Kuemmerle & Teng 2000
	Vascular smooth muscle cells/r	s	Gustafsson <i>et al.</i> 1999b
	Vascular smooth muscle cells/p	i	Duan & Clemmons 1998
	Vascular smooth muscle cells/h	i	Hayford <i>et al.</i> 1998
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	Fibroblasts/h	i	Conover <i>et al.</i> 1995
	Fibroblasts/b	s	Conover <i>et al.</i> 1995
	SH-SY5Y neuroblastoma cells/h	s	Babajko <i>et al.</i> 1997
IGF-II	Bone marrow stromal cells/h	i	Thomas <i>et al.</i> 1999
	Adrenocortical cells/b	s	Fottner <i>et al.</i> 1999
	TC3 pancreatic beta cells	n	Katz <i>et al.</i> 1997
	Non-small cell lung cancer cells	i	Fottner <i>et al.</i> 1999
	SH-SY5Y neuroblastoma cells/h	s	Babajko <i>et al.</i> 1997
IL-1 β	Ovarian granulosa cells	i	Chamoun <i>et al.</i> 1999
	Articular chondrocytes/h	i	Di Battista <i>et al.</i> 1997
	Fetal lung fibroblast/r	s	Price <i>et al.</i> 2002
IL-6	Hepatocytes/r	s	Fernandez-Celemin & Thissen 2001
	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
IFN- γ	Multiple myeloma/h	s	Feliers <i>et al.</i> 1999
Insulin	Ovarian thecal cells/b	n	Chamberlain & Spicer 2001
	L6 myoblasts/r	s	McCusker & Clemmons 1998
	TC3 pancreatic beta cells	n	Katz <i>et al.</i> 1997
	SH-SY5Y neuroblastoma cells/h	s	Babajko <i>et al.</i> 1997
	Granulosa cells/h	s	Greisen <i>et al.</i> 2002
Insulin+glucagon	Ovarian thecal cells/b	i	Chamberlain & Spicer 2001
N-myc oncogene	SK-N-SH neuroblastoma cells/h	s	Chambery <i>et al.</i> 1999
OP-1	Osteoblasts/r	i	Yeh <i>et al.</i> 1996

Continued

Table 2 Continued

	Cell types/species	Effect	Reference
Agent PDGF-BB	Fetal lung fibroblasts/r	s	Price 2001
	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
	Articular chondrocytes/h	s	Di Battista <i>et al.</i> 1997
	SaOS-2 osteoblasts/h	s	Kudo <i>et al.</i> 1996, 1997
PTH	SaOS-2 osteoblasts/h	s	Kudo <i>et al.</i> 1996, 1997; LaTour <i>et al.</i> 1990
	TE85 osteosarcoma cells/h	s	Honda <i>et al.</i> 1996
RA	Sertoli cells/p	s	Bardi <i>et al.</i> 1999
	Neuroblastoma cells/h	i	Chambery <i>et al.</i> 1998
	Osteoblasts/m	s	Glantschnig <i>et al.</i> 1996
	HT-29 colon carcinoma cells	s	Corkins <i>et al.</i> 2002
T ₃	Bone marrow stromal cells/r	s	Milne <i>et al.</i> 2001
	Sertoli cells/p	s	Bardi <i>et al.</i> 1999
	Primary hepatocytes/r	s	Demori <i>et al.</i> 1997a,b
	HepG2 hepatoma cells/h	n	Demori <i>et al.</i> 1997a
TGF- α	Osteoblasts/m	s	Glantschnig <i>et al.</i> 1996
	Granulosa cells/r	s	Piferrer <i>et al.</i> 1997
TGF- β 1	Glomerular endothelial cells/h	s	Giannini <i>et al.</i> 1999
	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
Thrombin	Stromal-vascular cells/p	s	Richardson <i>et al.</i> 1998
	Vascular smooth muscle cells/r	i	Anwar <i>et al.</i> 2000
TNF- α	Multiple myeloma/h	n	Feliers <i>et al.</i> 1999
	Fetal lung fibroblasts/r	s	Price <i>et al.</i> 2002
D ₃	Bone marrow stromal cells/h	s	Kveiborg <i>et al.</i> 2001
WT-1	Embryonic stem cells/m	s	Wagner <i>et al.</i> 2001

ACTH, adrenocorticotrophic hormone; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; EGF, epidermal growth factor; FSH, follicle-stimulating hormone; GH, growth hormone; IL, interleukin; IFN, interferon; OP-1, osteogenic protein-1; PDGF-BB, platelet-derived growth factor-BB; PTH, parathyroid hormone; RA, retinoic acid; T₃, triiodothyronine; TGF, transforming growth factor; TNF, tumor necrosis factor; D₃, 1,25-dihydroxyvitamin D₃; WT-1, Wilms tumor-1 protein; b, bovine; h, human; m, mouse; p, porcine; r, rat; s, stimulation; i, inhibition; n, no effect.

et al. 1998, Gustafsson *et al.* 1999a), B104 rat neuroblastoma cells (Cheung *et al.* 1991), HT-29 human colon adenocarcinoma cells (Culouscou & Shoyab 1991) and M12 human prostate cancer cells (Damon *et al.* 1998b). These inhibitory actions of IGFBP-4 have been demonstrated to be IGF-dependent on the basis of the following facts: (i) IGFBP-4 had no effect or lower potency in blocking the biological activity of IGF analogues which have significantly (>100-fold) reduced binding affinity to IGFBP-4 (Mohan *et al.* 1995b); and (ii) IGFBP-4 inhibited the binding of IGF-I to purified IGF-IR *in vitro* (Mohan *et al.* 1995b).

Consistent with the *in vitro* data, IGFBP-4 is also a functional antagonist of IGF actions *in vivo*. For example, transgenic mice overexpressing IGFBP-4 selectively in smooth muscle cells exhibits smooth muscle hypoplasia (Wang *et al.* 1998, Zhang *et al.* 2002), which is in direct contrast to the smooth muscle hypertrophy induced by IGF-I overexpression (Wang *et al.* 1997). Moreover, a protease-resistant IGFBP-4 had more potency (Zhang *et al.* 2002) and IGF-I/IGFBP-4 double transgenic mice showed a reduction in wet weight of selected smooth muscle tissues (Wang *et al.* 1998), suggesting that these inhibitory effects of IGFBP-4 are IGF-I-dependent. In

agreement with the above data, local administration of recombinant IGFBP-4 inhibited IGF-I-induced increases of bone formation in mice (Miyakoshi *et al.* 1999).

In contrast to local IGFBP-4 administration, systemic administration of IGFBP-4 increased bone formation (Miyakoshi *et al.* 1999, 2001). This was the only report regarding a growth-stimulatory effect of IGFBP-4, which was probably by increasing IGF bioavailability via an IGFBP-4 protease-dependent mechanism, since systemic administration of the native IGFBP-4, but not protease-resistant IGFBP-4, increased the levels of serum free IGF-I, serum osteocalcin, serum and skeletal alkaline phosphatase, and IGFBP-4 proteolytic activity in serum (Miyakoshi *et al.* 2001).

IGF-independent actions

In addition to IGF-dependent actions, IGF-independent actions of IGFBP-4 have been suggested, based on the following findings: (i) IGFBP-4 caused a marked inhibition of ceramide-induced apoptosis of Hs578T human breast cancer cells, which lack a functional IGF-IR (Perks *et al.* 1999); (ii) IGFBP-4 inhibited human ovarian steroidogenesis in the presence of either the IGF-IR blocker

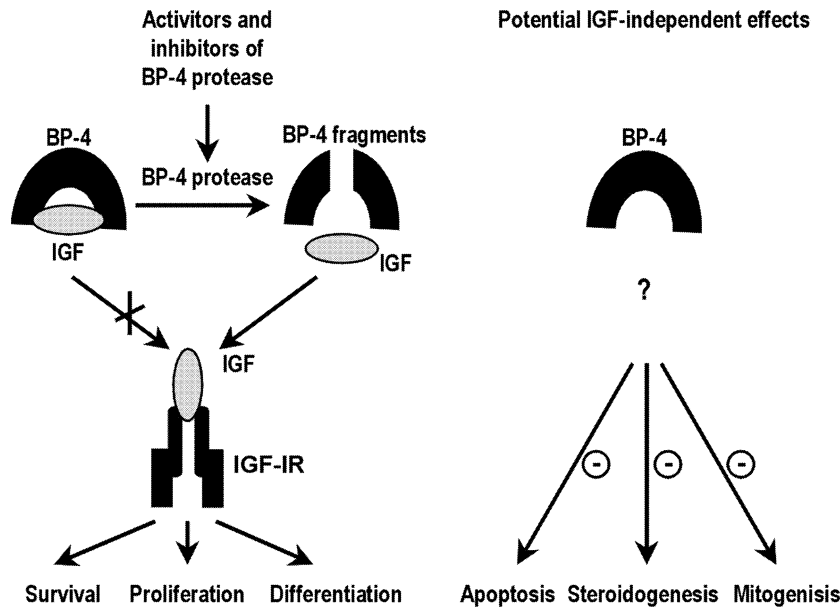


Figure 2 IGF-dependent and -independent actions of IGFBP-4 (BP-4). The mitogenic activity of IGFs, mediated by IGF-IR, is inhibited by the binding of IGFBP-4. Proteolysis of IGFBP-4 results in the release of IGFs from the IGF/IGFBP-4 complex and consequently a potentiation of IGF effects. Several potential IGF-independent effects of IGFBP-4 are also documented, but the mechanisms are unknown yet. Refer to the text for further details of these actions.

α IR3 or excess IGFBP-3 to remove the effects of endogenous IGF action (Wright *et al.* 2002); and (iii) endogenous IGFBP-4 inhibited the mitogenic effects of IGF and insulin in HT-29 human colonic adenocarcinoma cells, which could not be compensated for by the addition of an excess of IGF-I and insulin, but by the addition of an antibody against IGFBP-4 (Singh *et al.* 1994). However, unlike IGFBP-3 and -5, a specific receptor for IGFBP-4 has not been identified yet. Further studies are necessary to define the mechanisms of IGF-independent actions of IGFBP-4.

Biological significance of IGFBP-4

Reproductive physiology

The expression pattern of IGFBP-4 and its regulation were extensively studied in human and animal reproductive organs at various reproductive stages. The findings in these studies indicate important roles for IGFBP-4 in reproduction.

Pregnancy IGFs, as mitogenic peptides, are important for fetal and placental growth during pregnancy (Han & Carter 2000). In the human placenta, IGFs regulate syncytiotrophoblast steroidogenesis (Nestler 1987, 1990), glucose and amino acid transport in the villi (Kniss *et al.*

1994) and the invasion of the extravillous trophoblast into the maternal decidua (Han *et al.* 1996, McKinnon *et al.* 2001). IGFBPs are expressed by the maternal decidua and may regulate IGF actions during pregnancy (Han *et al.* 1996). In this context IGFBP-4 is particularly interesting for several reasons: (i) IGFBP-4 is the second most abundant IGFBP in the placental bed (Han *et al.* 1996); (ii) PAPP-A, an IGF-dependent IGFBP-4 protease, is secreted by human trophoblast cells and decidualized endometrial stroma (Giudice *et al.* 2002, Sun *et al.* 2002) and is markedly increased in the maternal circulation as pregnancy progresses (Byun *et al.* 2000); and (iii) in human pregnancy serum the majority of PAPP-A (>99%) is found as a PAPP-A/proMBP complex (Oxvig *et al.* 1993). During pregnancy, rapid placental development and fetal growth obviously increases the need for growth-promoting factors such as IGFs. The mitogenic activity of IGFs at the local cellular level depends on the concentration of free IGFs that are able to interact with their receptors. The increased IGFBP-4 proteolytic activity resulting from uncomplexed PAPP-A may be required locally to increase the concentration of free IGFs for placental development and therefore for fetal growth during pregnancy, whereas the PAPP-A activity in maternal circulation is inhibited by complex formation with proMBP. The absence of PAPP-A expression in the placenta of pregnant patients with Cornelia de Lange syndrome, a condition involving incomplete fetal development and subsequent deformities

(Westergaard *et al.* 1983), provides direct evidence for a role of PAPP-A in pregnancy. However, the roles of this complex system of enzyme (PAPP-A), substrate (IGFBP-4), inhibitor (proMBP) and cofactor (IGF-II) in the placenta and maternal circulation during human pregnancy deserve further investigation.

Different from human, recent data showed that the IGFBP-4 proteolytic activity in murine serum is not increased during pregnancy, eventually due to the lower level of PAPP-A expression in the placenta (Qin *et al.* 2002, Soe *et al.* 2002). The significance of this difference between species is unknown, but this difference must be taken into consideration when the mouse is used as a model organism for the study of PAPP-A function.

Ovarian physiology IGFs are produced by ovarian granulosa and theca cells, and mediate gonadotropin actions on ovarian cellular steroidogenesis and growth (Poretsky *et al.* 1999). Regulation of IGF actions within the ovarian follicle is particularly important in the processes of ovarian follicle development and follicle atresia (Monget *et al.* 2002). As a potent inhibitor of IGF actions, IGFBP-4 appears to be particularly important in ovarian physiology (Iwashita *et al.* 1996, Poretsky *et al.* 1999). High levels of IGFBP-4 are present in small androgen-dominant follicles (Zhou & Bondy 1993, el Roeiy *et al.* 1994) and in follicular fluid from androgen-dominant follicles (FF_a) that are growth-arrested or atretic (Cataldo & Giudice 1992, San Roman & Magoffin 1993). In contrast, IGFBP-4 is undetected by ligand blot analysis in follicular fluid from estrogen-dominant growing follicles (FF_e). IGFBP-4 inhibits human ovarian steroidogenesis *in vivo* (Mason *et al.* 1998, Wright *et al.* 2002), and it has been suggested that IGFBP-4 inhibits follicle development by inhibiting IGF actions in the ovary, and conversely, the loss of this inhibitory factor allows for increased bioavailable IGFs, which coincides with selection of the dominant follicle (Poretsky *et al.* 1999). The complexity of this process has become even more apparent with the finding of the IGFBP-4-specific protease PAPP-A activity in FF_e, but not in FF_a (Chandrasekher *et al.* 1995). PAPP-A is expressed in human and mouse ovaries, being restricted to healthy granulosa cells and granulosa-lutein cells (Hourvitz *et al.* 2000, 2002). This restricted expression pattern and its co-expression with aromatase and luteinizing hormone receptor in granulosa cells from preovulatory follicles suggests that PAPP-A could be considered as a functional marker of follicular development (Mazerbourg *et al.* 2001). During terminal development of follicles to the preovulatory stage, the degradation of IGFBP-4 by PAPP-A in the ovary may increase IGF bioavailability that further stimulates granulosa cell proliferation and steroidogenesis, and then actively participates in the selection of dominant follicles *in vivo*. In contrast, in atretic follicles, IGFBP-4 degradation is inhibited by locally increased IGFBP-2 and -5 that contain an HBD in their C-terminal

regions (Mazerbourg *et al.* 2000). The IGFBP-4 protease activity has been reported in human, ovine, bovine, porcine, equine (Mazerbourg *et al.* 2000, 2001) and murine ovaries (Hourvitz *et al.* 2002), suggesting a well-conserved process for this protease in ovarian function.

Bone formation

IGFs are the most abundant growth factors stored in the bone and regulate the proliferation and differentiation of bone cells (Bautista *et al.* 1990). As in other tissues, the local activity of IGFs in bone is modulated by IGFBPs. IGFBP-4 is one of the major IGFBPs produced by bone cells (Mohan *et al.* 1995b) and has been proposed to function as an important regulator of bone formation. Evidence was provided by the following findings: (i) IGFBP-4 inhibited both basal and IGF-induced cell proliferation of MC3T3-E1 mouse osteoblasts and untransformed normal human bone cells (Mohan *et al.* 1995b) and the growth of embryonic chicken pelvic cartilage *in vitro* (Schiltz *et al.* 1993); (ii) a single local administration of IGFBP-4 inhibited IGF-I-induced increases in bone formation, whereas systemic administration of IGFBP-4 alone increased serum and skeletal levels of bone formation markers (osteocalcin and alkaline phosphatase) in mice (Miyakoshi *et al.* 1999, 2001). The latter stimulatory effect resulted from an increase of IGF bioavailability in the circulation via an IGFBP-4 protease-dependent mechanism (Miyakoshi *et al.* 2001); (iii) the serum level of IGFBP-4 was shown to increase with aging and to correlate positively with serum PTH levels (Rajaram *et al.* 1997, Karasik *et al.* 2002). PTH upregulated IGFBP-4 expression in human osteoblasts *in vitro*, and serum IGFBP-4 levels were found to be increased during oral 1,25-dihydroxyvitamin D₃ therapy in psoriasis patients. These findings suggest that during calcium deficiency, the increase in serum PTH and 1,25-dihydroxyvitamin D₃ may not only stimulate bone resorption, but also inhibit bone formation by stimulating IGFBP-4 production in bone cells (Mohan *et al.* 1995a, Rajaram *et al.* 1997, Karasik *et al.* 2002); and (iv) sera from patients with chronic renal failure (CRF) usually contain high levels of IGFBP-4, which may contribute to decreased bone formation in renal osteodystrophy (Van Doorn *et al.* 2001).

Renal pathophysiology

IGFBP-4 is abundantly expressed in the kidney. A site-specific expression pattern of IGFBP-4 during nephrogenesis was described in the human (Matsell *et al.* 1994), rat (G J Price *et al.* 1995) and mouse (Lindenbergh-Kortleve *et al.* 1997), suggesting specific roles for IGFBP-4 in renal development and physiology. In addition, changes in IGFBP-4 abundance may be associated with pathological processes of the kidney. Upregulation of IGFBP-4 levels in serum correlated with the degree of renal

dysfunction and growth retardation of children with CRF (Uliniski *et al.* 2000, Van Doorn *et al.* 2001), while downregulation of renal IGFBP-4 expression was reported in growth hormone-induced rat hypersomatotrophy (Hise *et al.* 2001).

IGFBP-4 and cancer

IGFBP-4 is expressed in a range of cells of tumor origin, such as lung adenocarcinoma (W A Price *et al.* 1995), non-small-cell lung cancer (Noll *et al.* 1996), breast cancer (C Qin *et al.* 1999), colon carcinoma (Michell *et al.* 1997), follicular thyroid carcinoma (Bachrach *et al.* 1995), gastric cancer (Yi *et al.* 2001), glioma (Bradshaw *et al.* 1999), hepatoma (Scharf *et al.* 1998), myeloma (Feliars *et al.* 1999), neuroblastoma (Cheung *et al.* 1991, Babajko & Binoux 1996), osteosarcoma (Mohan *et al.* 1995b) and prostate cancer (Srinivasan *et al.* 1996, Damon *et al.* 1998b, Drivdahl *et al.* 2001).

In vitro and *in vivo* studies suggest that IGFBP-4 plays an important role in the growth regulation of a variety of tumors, possibly by inhibiting autocrine IGF actions or by as yet unknown IGF-independent mechanisms. Notably, in Caco-2 human colon carcinoma cells expression of IGFBP-4 mRNA was correlated with cell differentiation, indicating growth inhibitory effects in that cellular system (Hoeflich *et al.* 1996). Proliferation, anchorage-independent growth and tumor development in athymic nude mice were inhibited by overexpression of IGFBP-4 in M12 prostate cancer cells. Apoptosis was increased in the IGFBP-4-overexpressing cells, probably due to sequestering IGF ligands (Damon *et al.* 1998b). Blocking of IGFBP-4 with antibodies enhanced both basal and IGF-stimulated growth of HT-29 human colonic carcinoma cells in both an IGF-dependent and an IGF-independent manner (Singh *et al.* 1994). Recombinant IGFBP-4 caused marked inhibition of ceramide-induced apoptosis of Hs578T human breast cancer cells via an IGF-independent pathway (Perks *et al.* 1999).

Conclusions

IGFBP-4 is the smallest of the six high-affinity IGFBPs. It exists in both non-glycosylated and glycosylated forms in all biological fluids. IGFBP-4 is expressed by a large range of cell types and tissues, and its expression is affected by different mechanisms in a cell type-specific manner. IGFBP-4 binds IGF-I and IGF-II with similar affinities and inhibits their actions in almost all *in vitro* and *in vivo* conditions. IGF-independent actions have also been reported. Proteolysis is a major regulatory mechanism for IGFBP-4. An IGF-dependent IGFBP-4-specific protease has been identified, which plays important roles in regulating IGFBP-4 actions in reproductive physiology. In addition, IGFBP-4 is an important regulator for bone

formation and renal pathophysiology. Further studies are necessary to address the following questions: (i) Can IGFBP-4 enhance IGF actions? (ii) What is the mechanism underlying the IGF-independent actions of IGFBP-4? (iii) What is the subcellular distribution of IGFBP-4? (iv) What are the specific roles for IGFBP-4 *in vivo*? Biochemical, molecular biological and transgenic approaches will widen our understanding of the nature of this binding protein.

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